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(54) Title Of Invention: Chemically Modified Protein

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Patent Specification

1. Title of invention

Chemically Modified Protein

2. Patent claim

Chemically modified protein obtained by conjugating polyethylene glycol to the primary amino group of the islet-activating protein that was produced by bacteria of the Bordetella family.

3. Specifications

Fields of industrial application

The present invention deals with chemically modified protein.

Background

Whooping cough, namely pertussis, toxin is the toxin that is characteristically produced by bacteria of the Bordetella family, the causal bacteria of the whooping cough disease unique to humans.

This toxin is known to show various biological activities: for instance, it is known as histamine-sensitizing factor (HSF), leukocytosis-lymphocytosis-promoting factor (LPF), hemagglutinin (HA), mouse protective antigen (MPA), islet-activating protein (IAP), etc. Biological and protein chemistry investigations have been clarifying the real causal substance of the said activities and it is now generally accepted that LPF is a substance identical to IAP. (Pittman, M.: Rev. Infect. Dis. 1, 401-412 (1979).

The above mentioned islet-activating protein (could be abbreviated as IAP in the subsequent text) increases secretion of insulin and has pharmacological activities of adjusting and maintaining normal blood sugar level for an extended period in mammals. This suggested that the substance is useful as a preventive and therapeutic drug for diabetes and it has been investigated for this purpose. Consequently, a method is known for its manufacture and its physicochemical properties are also known. [Yajima, M; J. Biochem. 83, 295-303 (1978)]

Besides the said activities, however, it also shows side effects such as an increase in the white blood cell count, histamine sensitization, hemagglutination, etc. In addition, because it is a

protein of microbial origin, it is immunogenic as a foreign protein. Therefore, development of a substance having only islet-activating activity with little or no side effects has been looked forward to.

To this end, chemical modification of IAP had been attempted [Nogimori et al.: Biochim. Biophys. Acta, 801, 220-231 (1984); ibid. 801, 232-243 (1984)], but the reduction of side effects, especially lowering of antigenicity and immunogenicity, could not be expected.

Problems that the present invention intends to solve

The present invention is to develop IAP derivatives which retain islets activating activities with lessened side effects.

Ways to solve the problems

The present invention is to offer the chemically modified protein that is formed by conjugating polyethylene glycol to the primary amino group of islet-activating protein produced by bacteria of the Bordetella family.

The above described IAP, which is produced by bacteria of the Bordetella family, could be either in the purified or semi-purified form as long as it shows islet-activating properties. Examples of such preparations include those described in the reference for the above-mentioned IAP production or obtained according to the following reference examples.

For the primary amino group of the protein, examples are ε-amino group of lysine and the primary amino group at the amino-terminal of the protein.

The polyethylene glycol residue conjugated for the above-mentioned purpose may be expressed by the following formula.

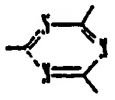
$$-O-(CH_2CH_2O)_l-R$$

[where R indicates a group protecting the hydroxyl group. l represents an integer within the range of 7 ~ 700.]

For the chemically modified protein of the present invention, the above-mentioned polyethylene glycol is desirably conjugated to the said primary amino group via a spacer.

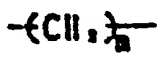
This spacer could be any kind as long as it mediates the bonding between the said primary amino group and polyethylene glycol. Examples are:

i) Chemical group expressed by the formula



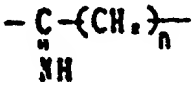
(triazine).

ii) Chemical group expressed by the formula



[where m represents an integer between 1 ~ 3] (alkylene).

iii) Chemical group expressed by the formula



[where n represents an integer between 1 ~ 3] (imidate).

With regards to the said spacer, in the case of triazine, the spacer may have 1 or 2 polyethylene glycol molecules; especially, those having 2 molecules of polyethylene glycol are favorable. For the alkylene spacer and the imidate spacer, the more desirable ones are those with m or n being the integer 2.

For the chemically modified protein of the present invention, each IAP-molecule is preferably conjugated with roughly 1 ~ 30 molecules of polyethylene glycol or spacer with polyethylene glycol, with the especially desirable range being 1 ~ 10 molecules. Also, roughly 3 ~ 80%, more desirably roughly 3 ~ 40%, of the primary amino groups in an IAP molecule are preferably conjugated with the said polyethylene glycol or the spacer with polyethylene glycol.

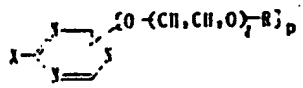
With regards to the above-mentioned polyethylene glycol, the group R protecting the hydroxyl group is a lower alkyl (for instance, 1~3 carbon atoms) or a lower alkanoyl (for instance, 1~3 carbon atoms). Examples of the former include methyl, ethyl, propyl, i-propyl and those of the later are formyl, acetyl, propionyl. The methyl group is especially favorable as the group protecting the hydroxyl group. For *l*, the especially desirable range is about 80 ~ 300; that

is, the mean molecular weight of the polyethylene glycol is in the range of roughly 350 ~ 30000, with the more desirable one being roughly 1900 ~ 15000.

The chemically modified protein of the present invention could be prepared by bringing the islet-activating protein (IAP) which was produced by bacteria of the Bordetella family to react with the compound having polyethylene glycol (activated PEG).

The following is a description of the manufacturing method for the chemically modified protein of the present invention.

The chemically modified protein of the present invention with triazine being the spacer could be prepared by bringing IAP to react with the compound expressed by the formula,

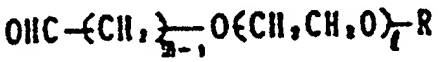


Formula (I)

[where: R and *l* represent the same as those described above. X is a halogen and p indicates an integer of 1 or 2.]. In this case, X is desirably chlorine.

The reaction was performed in aqueous buffer solution such as phosphate buffer, borate buffer, etc., at a pH of about 8 ~ 10, temperature 0°C ~ room temperature for 1 ~ 24 hours. For the reaction, 1 ~ 500 times the amount in moles., more preferably 5 ~ 200 times the amount in moles of the compound (I) was used relative to IAP.

In the case of chemically modified protein of the present invention, where the spacer is alkylene, the protein could be obtained by the reaction between IAP and the compound expressed by the following formula,



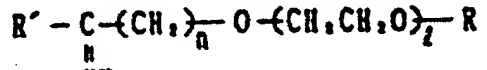
Formula (II)

[where R, m and *l* represent the same as those described above.],
in the presence of a reducing agent.

The reaction was carried out in an aqueous buffer solution, such as phosphate buffer, borate buffer, etc. at a pH about 6.0 ~ 9.0, temperature 0°C ~ 50°C for 10 ~ 80 hours. For the reducing

agent, a boron-type reducing agent may be used advantageously, and examples are sodium borohydride, sodium cyanoborohydride, etc. For the reaction, 1 ~ 1000 times the amount in moles, more preferably 5 ~ 200 times the amount in moles of the compound (II) and 1 ~ 100 times the amount in moles of the reducing agent were used relative to IAP.

In the case of the chemically modified protein of the present invention where the spacer is imidate, the protein could be prepared by the reaction between IAP and the compound expressed by the following formula,



Formula (III)

[where R, n and *l* represent the same as those described above. R' indicates a lower alkoxy].

For R', the desirable examples are alkoxy having 1 ~ 3 carbon atoms (examples: methoxy, ethoxy, propoxy). In addition, the compound (III) may be used as a salt, such as hydrochloride, sulfate, acetate, etc.

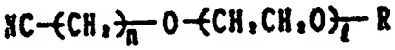
The reaction was carried out in aqueous buffer solution, such as phosphate buffer, borate buffer, etc., at a weakly alkaline pH of about 7.0 ~ 9.0, temperature 0° C ~ 40°C for 3 ~ 30 hours.

The proportion of modified amino groups depends upon the range of the said activated PEG amount used in the reaction and may be varied freely according to the amount of the latter. A desirable amount of its use is roughly 5 ~ 1000 mol, with the especially desirable range being roughly 50 ~ 500 mol per 1 mol of IAP in the case of a triazine spacer; while, for conjugation via imidate or alkylene spacer, a desirable amount of its use is roughly 10 ~ 2000 mol, with the especially desirable range being roughly 50 ~ 1000 mol, per mol of IAP.

To obtain the intended chemically modified protein, the reaction mixture may be purified by means of methods usually employed for protein purification according to one's choice, such as dialysis, salting out, ultrafiltration, ion-exchange chromatography, gel filtration, high performance liquid chromatography, electrophoresis, etc. Notably, ultrafiltration and gel filtration methods are very effective in removing unreacted PEG. The proportion of modified amino groups may be computed, for instance, by amino acid analysis after acid decomposition [of the modified protein --- Translator].

The raw materials, namely compounds (I) and (II), are well known materials and the preparation methods and physicochemical properties of these compounds have been published, for instance, in Chemistry Letters, 773 (1980); EPC KOKAI No.154316 KOHO.

Also, compound (III) could be prepared by hydrolysis of the known material having the following formula



Formula (IV)

[where R, n and *l* represent the same as those defined above.]
using a usual method in the presence of lower alkanol (methanol, ethanol, propanol, etc.) and acid (hydrogen chloride, hydrochloric acid, sulfuric acid, acetic acid, etc.).

The chemically modified protein of the present invention has strong islets-activating activity but with markedly reduced side effects of unmodified IAP, such as leukocytosis-lymphocytosis-promoting factor activity, histamine sensitization and hemagglutination.

Also, the chemically modified protein of the present invention shows markedly lower antigenicity and immunogenicity, neither of which was possible to achieve with known unmodified IAP and its related materials. In addition, the modified protein exhibits low toxicity.

Therefore, the chemically modified protein of the present invention is extremely useful as a preventive and therapeutic drug for diabetes in mammals (rat, mouse, dog, cat, human, etc.).

For example, when the chemically modified protein of the present invention is used as a therapeutic drug for diabetics, its dose per day for an adult person would be 10 ng/kg ~ 500 μg/kg for injection and 1 Mg/kg ~ 500 mg/kg for oral drug preparation.

Actions

The actions of the chemically modified protein of the present invention can be recognized from the following experimental examples.

Experimental Example 1 Islet-activating activity

Separately dissolved unmodified IAP and PEG-IAP obtained in Practical Examples 1 ~ 4 in saline and 1 mL (4 μg as protein) of the respective solution was injected into SD rats (male, each group consisting of 5 animals) via vein in the tail. After 3 days, variations in blood sugar and blood insulin level responding to sugar loading were investigated. In this case, the animals were fasted for 24 hours prior to the beginning of the experiment. Immediately after collecting 0.1 mL of blood from the vein in rat tail, 1 mL per 100 g bodyweight of 20% glucose solution was injected into the intraperitoneal cavity of the experimental animal. Then, 0.1 mL of blood was withdrawn for testing at 15, 30 and 60 minutes after the glucose injection. The blood sugar level was determined by means of the glucose oxidase method and the insulin was measured using the double antibody method. The results shown in Tables 1 ~ 3 clearly indicate a drop in the blood sugar level and increased insulin in the blood of the animals in both groups treated with either IAP or PEG-IAP, suggesting islet-activating activity of the PEG modified protein.

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Table 1. Variations of blood sugar level and insulin in the blood after sugar loading to rats that had been injected with PEG-IAP obtained in Practical Example 1.

		MW of PEG	Time after administration of glucose (minute)			
			0	15	30	60
Blood sugar level (mg/dL)	Control	--	72.0 ± 5.0	196.0 ± 13.7	169.4 ± 13.8	110.0 ± 11.1
	Unmodified IAP	--	69.8 ± 5.7	176.3 ± 31.6	*115.8 ± 14.6	108.0 ± 13.6
	PEG-IAP	5000	67.5 ± 5.1	189.5 ±18.8	*118.8 ± 20.9	114.3 ± 7.8
	PEG-IAP	1900	69.8 ± 6.5	*169.0 ± 33.5	147.2 ± 36.9	97.0 ± 10.9
	PEG-IAP	750	65.3 ± 4.9	165.5 ± 16.8	145.0 ± 26.2	103.3 ± 4.8
	PEG-IAP	350	*62.8 ± 4.3	185.5 ± 26.2	157.0 ± 18.5	102.0 ± 8.0
Insulin level (μU/mL)	Control	--	1.5 ± 3.2	38.2 ± 47.3	42.1 ± 29.7	21.2 ± 10.1
	Unmodified IAP	--	1.5 ± 1.9	*136.3 ± 75.0	**308.2 ± 104.0	*160.0 ± 127.1
	PEG-IAP	5000	1.8 ± 3.2	188.0 ± 141.4	*164.3 ± 98.9	74.7 ± 98.0
	PEG-IAP	1900	3.8 ± 3.7	*177.2 ± 81.6	*144.6 ± 6.6	38.8 ± 12.6
	PEG-IAP	750	3.7 ± 2.3	170.7 ± 109.7	6.95 ± 52.4	27.7 ± 13.8
	PEG-IAP	350	5.5 ± 3.9	*201.0 ± 122.3	** 265.4 ± 113.7	8.3 ± 9.1

Mean ± standard deviation *: P<0.05 **: P<0.01

Table 2. Variations of blood sugar level and insulin in the blood after sugar loading to rats that had been injected with PEG-IAP obtained in Practical Example 2.

			Time after administration of glucose (minute)			
		Molar Ratio	0	15	30	60
Blood sugar level (mg/dL)	Control		68.6 ± 10.0	177.0 ± 36.8	175.8 ± 26.5	126.2 ± 21.4
	Unmodified IAP		70.8 ± 5.4	157.6 ± 22.9	**138.5 ± 14.6	**92.4 ± 7.4
	PEG - IAP	10	75.4 ± 5.9	202.6 ± 29.0	**126.6 ± 26.0	**94.6 ± 5.3
	PEG - IAP	50	73.8 ± 6.1	211.4 ± 45.7	158.2 ± 30.7	**94.2 ± 7.4
	PEG - IAP	200	*60.8 ± 4.6	163.2 ± 38.6	*142.5 ± 27.6	**98.3 ± 9.6
Insulin level (μU/mL)	Control		5.9 ± 1.3	29.5 ± 14.0	26.1 ± 10.9	11.8 ± 5.4
	Unmodified IAP		23.9 ± 30.6	**106.8 ± 23.3	**63.6 ± 33.1	**21.0 ± 8.4
	PEG - IAP	10	**11.7 ± 3.2	*69.5 ± 32.4	32.0 ± 13.9	10.4 ± 1.6
	PEG - IAP	50	*8.3 ± 2.0	**76.2 ± 23.4	34.7 ± 8.8	12.9 ± 3.0
	PEG - IAP	200	6.4 ± 2.9	*60.6 ± 28.9	21.8 ± 12.5	13.0 ± 6.0

Mean ± standard deviation *: P<0.05 **: P<0.01

'PEG/IAP molar ratio at the time of PEG-IAP preparation (the same in the subsequent tables)

Table 3. Variations of blood sugar level and insulin in the blood after sugar loading to rats which had been injected with PEG-IAP obtained in Practical Example 3 or 4.

			Time after administration of glucose (minute)			
		Preparation Method	0	15	30	60
Blood sugar level (mg/dL)	Control	--	75.0 ± 13.3	220.7 ± 30.4	179.5 ± 27.1	119.0 ± 17.3
	Unmodified IAP	--	80.3 ± 12.5	*271.5 ± 44.5	179.5 ± 33.5	*95.3 ± 11.2
	PEG - IAP	Practical Example 3	74.5 ± 8.2	274.0 ± 53.2	168.8 ± 36.2	*86.8 ± 17.7
	PEG - IAP	Practical Example 4	84.8 ± 6.9	226.3 ± 41.2	*140.0 ± 23.5	110.3 ± 4.4
Insulin level (μU/mL)	Control	--	9.4 ± 3.9	109.5 ± 50.0	56.3 ± 36.4	20.5 ± 6.8
	Unmodified IAP	--	12.9 ± 10.9	358.2 ± 288.0	**258.8 ± 44.1	**119.7 ± 72.5
	PEG - IAP	Practical Example 3	35.1 ± 22.0	**312.1 ± 90.8	266.5 ± 211.7	133.9 ± 101.3
	PEG - IAP	Practical Example 4	28.5 ± 27.6	223.3 ± 61.5	34.9 ± 281.2	140.7 ± 128.7

Mean ± standard deviation *: P<0.05 **: P<0.01

Experimental Example 2 Leukocytosis-lymphocytosis-promoting factor action (ΔLPF activity)

Each of A/J mice (male) was intravenously injected with 0.4 μg (as protein) of either unmodified IAP or PEG-IAP obtained in Practical Example 2. Five (5) days after the injection, the white blood cell count was determined and compared with that of the control group. The difference was taken as the increase in the white blood cell count.

ΔLPF activity = (white blood cell count of drug treated group) -
(white blood cell count of the control group)

The results shown in Table 4 clearly indicate that PEG-IAP had no or reduced leukocytosis-lymphocytosis-promoting factor activity.

Table 4. Leukocytosis-lymphocytosis-promoting factor activity

	Molar Ratio	ΔLPF activity (x $10^3/mm^3$)
Unmodified IAP		72
PEG - IAP	10	11
PEG - IAP	50	0
PEG - IAP	200	0

Experimental Example 3 Histamine sensitizing factor action

Each of A/J mice (male, each group comprising 10 animals) was intravenously injected with 2μg of either unmodified IAP or PEG-IAP obtained in Practical Example 2. Four (4) days after the injection, each animal was further injected with 2.5 mg histamine into the peritoneal cavity. The number of animals which died within 1 hour after the injection was expressed as histamine sensitizing factor action and the results are indicated in Table 5. The histamine sensitizing factor activity is eliminated in the case of PEG-IAP.

Table 5. Histamine sensitizing factor activity

	Molar Ratio	Histamine sensitizing activity
Unmodified IAP		10
PEG-IAP	10	0
PEG-IAP	50	0
PEG-IAP	200	0

Experimental Example 4 Hemagglutination factor activity

Unmodified IAP and PEG-IAP obtained in Practical Example 2 were separately and serially diluted with 10 mM phosphate buffer, pH 7, containing 0.15 M sodium chloride. The diluted solutions were placed into the U-shaped bottom multiwell plate so that each well contained 50 μL of a respective diluted solution, followed by adding 50 μL of 0.6% goose red blood cell (product of Japan Biological Material Center) suspended in the same buffer into each

well and mixing. After allowing the plate to stand at room temperature for 2 hours, the presence of red corpuscle agglutination was visually examined. The results shown in Table 6 indicate that PEG-IAP has a reduced hemagglutination factor activity.

Table 6. Hemagglutination factor activity

		Protein Concentration (μg/mL)							
	Molar Ratio	25	12.5	6.3	3.1	1.6	0.78	0.39	0.21
Unmodified IAP		+	+	+	+	+	+	--	--
PEG-IAP	50	+	+	+	--	--	--	--	--

Hemagglutination: +: present --: none

Experimental Example 5 Antigenicity

The antigenicity was determined by means of enzyme immunoassay (sandwich method). The antibody to IAP was obtained by immunizing goats and isolating anti-IAP-IgG using affinity purification. Alkaline phosphatase labeled anti-IAP-IgG was prepared by conjugating alkaline phosphatase (Miles Lab.) to the anti-IAP-IgG obtained above, using the glutaraldehyde method. The following is a detailed description of procedures for the enzyme immunoassay method.

The anti-IAP-IgG was dissolved in 50 mM carbonate buffer (pH9.7) (0.1 μg/mL) and placed into 96 well microplate (Maker: Nunk[1], Danish) so that each well contains 100 μL. The plate was left at 4°C overnight to allow the antibodies bind to the plate. After rinsing the anti-IAP-IgG bound plate with 10mM phosphate buffer, pH 7.4, containing 0.14M sodium chloride, 3 mM potassium chloride and 0.05% Tween 20, 100 μL of either unmodified IAP or PEG-IAP (0 ~ 200 ng/mL) obtained in Practical Examples 1~4, which had been diluted with the same buffer, was introduced into each well and was allowed to stand at room temperature for 2 hours. After rinsing the plate with the same buffer, 100 μL of alkaline phosphatase labeled anti-IAP-IgG (roughly 0.25 μg/mL), which had been dissolved in the same buffer, was added to each well and was allowed to stand at room temperature for 2 hours. After rinsing with the same buffer, the alkaline phosphatase activity was determined. For determination of the alkaline phosphatase activity, 200 μL of p-nitrophenyl phosphate (1 mg/mL), dissolved in 1 M diethanolamine buffer, pH 9.8, containing 0.01% magnesium chloride hexahydrate was added to the well and the enzyme was allowed to react at room temperature for 1 hour. Then, the increase in its light absorption at a wavelength of 405 nm was measured using a MTP-12 type microplate photometer (Manufacturer: Corona Co.). The results are shown in Table 7. The antigenicity

[1] Phonetic translation.

was expressed by light absorption ratio in % relative to that of unmodified IAP using the unmodified IAP light absorption as 100%.

Table 7. Antigenicity

	Preparation Method	MW of PEG	Proportion of modified amino groups (%)	Antigenicity (%)
Unmodified IAP		--	--	100
PEG-IAP	Practical Example 1	350	22.5	76
PEG-IAP	Practical Example 1	750	30.5	59
PEG-IAP	Practical Example 1	1900	15.8	60
PEG-IAP	Practical Example 1	5000	16.7	40
PEG-IAP	Practical Example 2	5000	3.0	60
PEG-IAP	Practical Example 2	5000	19.7	38
PEG-IAP	Practical Example 2	5000	35.8	10
PEG-IAP	Practical Example 3	5000	37.0	78
PEG-IAP	Practical Example 4	5000	16.4	38

Experimental Example 6 Immunogenicity

Determination of immunogenicity was carried out according to the method published in the Journal of Immunological Methods 14, 381 (1977). After emulsifying unmodified IAP or PEG-IAP obtained in Practical Example 2 (2 μg as protein) using Freund's complete adjuvant (FCA), it was injected into the peritoneal cavity of A/J mouse (each group consisting of 8 animals) and booster shots were administered on the14th and 28th day. Beginning from the14th day, blood was collected from the orbital vein of mouse every 7th day and its serum was evaluated for antibody production by means of the passive cutaneous anaphylaxis (PCA) reaction, using rats. Pre-diluted serum 0.1 mL was injected into the skin of the animal and, 4 hours later, 2 mL of 100 μg unmodified IAP and 20 mg Evans blue mixture solution was injected intravenously into the same animal. The reaction was judged by the permeability of blood vessels to the blue dye. The results are listed in Table 8. The number in the Table represents the maximum dilution of the PCA reaction positive serum. The anti-unmodified IAP antibody production was observed in the cases of unmodified IAP, PEG-IAP 10 and PEG-IAP 50 but not for PEG-IAP 200.

Table 8. Immunogenicity

	Molar ratio	day of PCA titer determination			
		14	21	28	35
Unmodified IAP		--*	--	--	16
PEG-IAP	10	--	--	4	32
PEG-IAP	50	--	--	--	16
PEG-IAP	200	--	--	--	--

*: < 4

Experimental Example 7 Improvement of glucose tolerance in diabetes model rats

Diabetes model rats were produced by subcutaneously injecting 120 mg/kg streptozocin into each of 1.5-day old female Wister Kyoto rats and continuing to rear them up to 8 weeks of age. The glucose tolerance of the animals was determined 6 days after intravenous injection of 2 μg of either unmodified IAP or PEG-IAP obtained in Practical Example 2. In addition, the bodyweight and WBC were measured 5 and 11 days after applying the dose. The glucose tolerance determination was performed as follows. Blood, 0.1 mL, was collected from the vein in the tail of a model rat that had been fasted for 24 hours just prior to the start of the test. This was immediately followed by oral administration of glucose (2 g/kg) and 0.1 mL of blood was collected at each subsequent time point of 15, 30, 60 and 120 minutes. The plasma glucose level of the collected blood sample was determined using the glucose oxidase method. Plasma glucose level, bodyweight and WBC are indicated in Fig. 1, Fig. 2 and Fig. 3 respectively.

As shown in Fig.1, diabetic rats indicate considerable deterioration of glucose tolerance relative to those of normal rats; while animals in the groups administered either unmodified IAP or PEG-IAP show improved glucose tolerance, similar to that of normal rat. Also, the unmodified IAP-treated group showed a bodyweight decrease and had increased WBC up to 3 times of the control group. On the other hand, the PEG-IAP treated group did not show any significantly different body weight and WBC compared to those of the control group.

The above-described results clearly suggest that chemical modification of IAP with PEG eliminates side effects, namely bodyweight loss and WBC increase, while retaining the glucose-tolerance-improving activity of unmodified IAP.

Practical Examples

The following is a more practical description of the present invention by means of reference examples and practical examples.

Reference Example 1 Preparation of IAP

The fraction having a low endotoxin and a high chicken red blood cell agglutination value (HA-titer), which had been obtained through a treatment at 100°C for 3 minutes according to the method proposed in EPC KOKAI No.47802 of Practical Example 1, was passed through a hydroxyapatite column that had been equilibrated to pH 8.0 to remove the FHA. The fraction which passed through was adjusted to pH 6.0 with hydrochloric acid and further passed through a new hydroxyapatite column which had been equilibrated to pH 6.0. The adsorbed crude IAP was eluted with 0.1 M phosphate buffer (pH 7.0) containing 0.5 M sodium chloride and then passed through an anti-FHA conjugated Sephadex column. Finally, IAP was isolated using sucrose density gradient centrifugation. The obtained IAP was used in the Practical Examples.

Reference Example 2 Synthesis of 2,4-bis(O-polyethylene glycol methyl ether)-6-chloro-S-triazine

Cyanuric chloride, 730 mg, was added to a mixture of 40 g polyethylene glycol methyl ether (mean MW 5000), 200 mL benzene, 20 g anhydrous sodium carbonate and 10 g molecular sieve 3A (WAKO Pure Chemicals Industries, K.K.) and made to react at 80°C for 20 hours with constant stirring. Upon completion of the reaction, 400 mL of petroleum ether was added to precipitated 2,4-bis(O-polyethylene glycol methyl ether)-6-chloro-S-triazine. Then, the precipitate was dissolved in benzene. This precipitating and dissolving cycle was repeated 3 times to remove the unreacted cyanuric chloride. Finally, the precipitate was dried in a desiccator under vacuum and 36 g 2,4-bis(O-polyethylene glycol methyl ether)-6-chloro-S-triazine was obtained . The determination by means of Maricle's method (Anal. Chem. 35, 683(1963)) revealed that the chlorine content of this compound was 0.32% and this roughly agreed with the theoretical value of 0.35%.

Polyethylene glycol methyl ether samples having a mean molecular weight of 350, 750 or 1900, were processed through the same procedure and the corresponding 2,4-bis(O-polyethylene glycol methyl ether)-6-chloro-S-triazines were obtained.

Yield of each corresponding compounds was 10.0 g, 13.2 g and 33.6 g.

The chlorine content of the obtained compound was 7.5%, 4.2% and 1.5% respectively and these values are in fairly good agreement with the respective theoretical values of 7.6%, 4.1% and 1.7%. Those with a mean molecular weight of 350 and 750 had a highly viscous form at normal ambient temperature.

Reference Example 3 Synthesis of polyethylene glycol monomethyl ether aldehyde

i) Polyethylene glycol methyl ether (5 g, mean MW 5000) was dissolved in methylene chloride (100 mL), followed by the addition of pyridinium chlorochromate (330 mg). The mixture was stirred at room temperature for 12 hours and the reaction mixture was diluted with a twofold volume of methylene chloride. The diluted reaction mixture was applied to a column of Florisil (Serva[2] Co., West Germany) (6 x 10 cm) and the column was washed with methylene chloride followed by chloroform. Then, it was eluted with methanol-chloroform (1:9). The fractions showing a positive reaction in the 2,4-dinitrophenylhydrazine test were collected, the solvent was removed by evaporation in vacuum and the intended compound was obtained as a crystalline waxy substance. The yield was 1.5 g (30%). Thin-layer chromatography: Rf = 0.08 (chloroform : ethanol : acetic acid = 9 : 1 : 0.5, silica gel) The aldehyde group was detected as the hydrated type

$$(-\underset{=}{C}H(OH)_2)$$

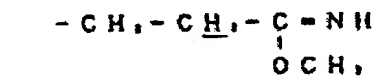
at 96.2PPM by ^{13}C-NMR.

Reference Example 4 Synthesis of polyethylene glycol imidoester form

Two grams (2 g) of polyethylene glycol methyl ether mono-β-cyanoethyl ether, synthesized from polyethylene glycol methyl ether having a mean MW of 5000, was dissolved in 15 mL of absolute methanol and dry hydrogen chloride was blown into it until saturation at a temperature of -20°C or lower. After it was tightly sealed and was allowed to stand in a freezer for 3 days, anhydrous ether was added to it and again was allowed to stand undisturbed in the freezer. Four (4) hours later, the upper ether layer was decanted, replaced with fresh anhydrous ether, mixed well, and the mixture was allowed to stand in the freezer. Roughly 1 hour after this, a solid material was formed. The solid material was washed well by removing the ether layer, adding new anhydrous ether to it, placing it in the freezer, and discarding the ether when the precipitation was completed. This cycle of processes was repeated 2 more times to wash the solid material very well. Then, the obtained solid material was dried under vacuum in a

[2] Phonetic translation.

desiccator with phosphorus pentoxide and solid NaOH for 1 hour. The obtained substance was polyethylene glycol imidoester having a mean MW of 5000. The yield was 1.5g and the triplet attributable to



was observed at the position δ 2.3 by NMR ($d_{illegible}$ - in DMSO, 90MHz). In addition, the absorption attributable to -CN disappeared in the IR.

Practical Example 1 Preparation of PEG-IAP

To 2.5 mg IAP, 2,4-bis(O-polyethylene glycol methyl ether)-6-chloro-S-triazine obtained in Reference Example 2 and having a mean MW of either 350 or 750 or 1900 or 5000 was added, so that the latter was present at a molar ratio 200 times relative to the former. The reaction was carried out in 25 mL of 0.1 M borate buffer, pH 9.0, at 4°C for 2 hours and was terminated by adding 25 mL of 0.2 M phosphate buffer, pH 7, to the reaction mixture. PEG-IAP was obtained by removing the unreacted PEG from the reaction mixture using ultrafiltration (Amicon (U.S.A.), PM-30 membrane) and purifying 2.0 mL of the concentrated reaction mixture by means of gel filtration through a Sephacryl S-200 (Pharmacia, Sweden) column (1.8 x 77 cm). The amount of protein was determined by Lowry's method. The extent of amino group modification was computed from the proportion of free amino groups determined using the fluorescamine method [Arch. Biochem. Biophys. 155, 213-220 (1973)]. The results are listed in Table 9.

Table 9. Proportion of modified amino groups in PEG-IAP

	MW of PEG	Proportion of modified amino groups (%)
PEG-IAP	350	22.5
PEG-IAP	750	30.5
PEG-IAP	1900	15.8
PEG-IAP	5000	16.7

Practical Example 2 Preparation of PEG-IAP

To 1 mg IAP, 2,4-bis(O-polyethylene glycol methyl ether)-6-chloro-S-triazine obtained in Reference Example 2 and having a mean MW of 5000 was added so that the latter was present at a molar ratio 10 or 50 or 200 times relative to the former. The reaction was carried out in 4.0 mL of 0.1 M borate buffer, pH 9, at 4°C for 2 hours and terminated by adding 4.0 mL of 0.1 M phosphate buffer, pH 7, to the reaction mixture. PEG-IAP was obtained by removing the

unreacted PEG from the reaction mixture using ultrafiltration (Amicon (U.S.A.), PM-30 membrane) and purifying 2.0 mL of the concentrated reaction mixture by means of gel filtration through a Sephacryl S-200 (Pharmacia, Sweden) column (1.8 x 77 cm). The amount of protein was determined by Lowry's method. The extent of amino group modification was computed from the proportion of free amino groups determined using fluorescamine method. The results are listed in Table 10.

Table 10. Proportion of modified amino groups in PEG-IAP

	Molar ratio	Proportion of modified amino groups (%)
PEG-IAP	10	3.0
PEG-IAP	50	19.7
PEG-IAP	200	35.8

Practical Example 3 Preparation of PEG-IAP

To 2 mg IAP, polyethylene glycol methyl ether aldehyde, obtained in Reference Example 3, was added so that the latter became 1000 times the molar ratio relative to the former. The reaction was performed in 2 mL of 0.1 M phosphate buffer, pH 7.0, containing 0.2 M urea at room temperature for 30 minutes. Then, 50 μL of pyridineborane, which had been dissolved in methanol (55 mg/mL), was added to the reaction mixture and the reaction was continued at room temperature for 2 more hours. The reaction was terminated by adding 2 mL of 1 M glycine. PEG-IAP was obtained by removing the unreacted PEG from the reaction mixture using ultrafiltration (Amicon (U.S.A.), PM-30 membrane) and purifying 2.0 mL of the concentrated reaction mixture by means of gel filtration through a Sephacryl S-200 (Pharmacia, Sweden) column (1.8 x 77cm). The proportion of modified amino groups determined using the fluorescamine method was 37.0 %.

Practical Example 4 Preparation of PEG-IAP

To 2 mg IAP, polyethylene glycol imidoester obtained in Reference Example 4 was added, so that the latter was present at 2000 times the molar ratio relative to the former. The reaction was carried out in 2 mL of 0.1 M phosphate buffer, pH 7.0, containing 2M urea at 4°C for 2 hours and terminated by addition of 40 mL of 1 M ammonium acetate, pH 6.0. PEG-IAP was obtained by removing unreacted PEG from the reaction mixture using ultrafiltration (Amicon (U.S.A.), PM-30 membrane) and purifying 2.0 mL of the concentrated reaction mixture by means of gel filtration through a Sephacryl S-200 (Pharmacia, Sweden) column (1.8 x 77cm). The proportion of modified amino groups determined using the fluorescamine method was 16.4 %.

Effects of the invention

The chemically modified protein of the present invention shows a strong islet-activating activity while exhibiting a lesser degree of the various side effects that unmodified IAP produces; therefore, it is useful as a preventive and therapeutic drug against diabetes.

4. Figure legends

Fig. 1, 2 and 3 illustrate glucose tolerance (plasma glucose level), bodyweight changes and WBC of diabetic rats indicated in Experimental Example 7, respectively. In Fig. 1, -o-, -x-, -●- and -Δ- represent control, normal rat, IAP and PEG-IAP rat group, respectively. In Fig.2, -o-, -x-, -●- and -Δ- represent control, normal rat, IAP and PEG-IAP rat group, respectively.

Agent Attorney Hiroshi Iwata

Figure 1

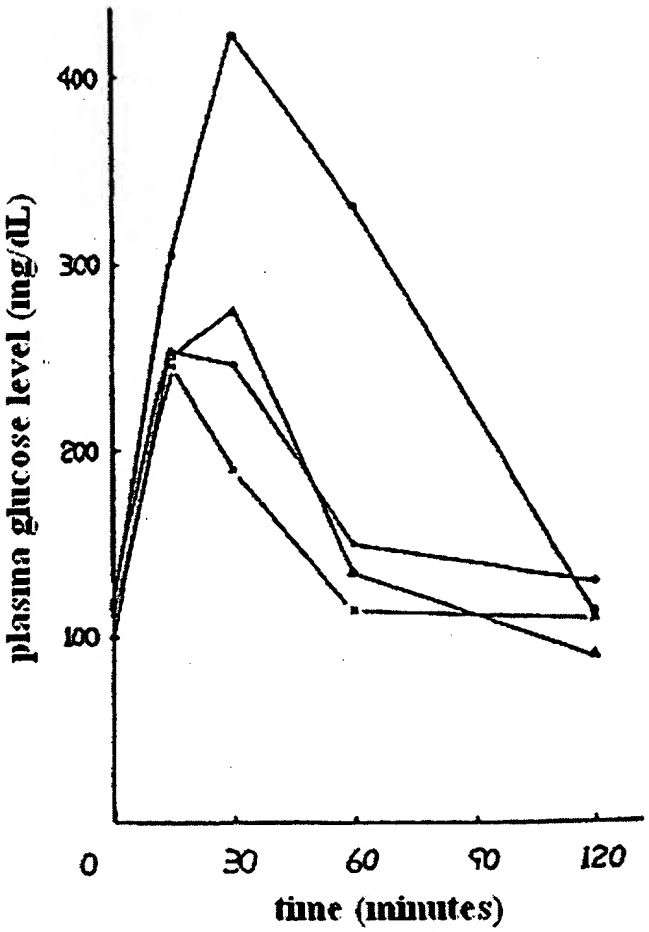

400
300
200
100
plasma glucose level (mg/dL)
0
30
60
90
120
time (minutes)

Figure 2

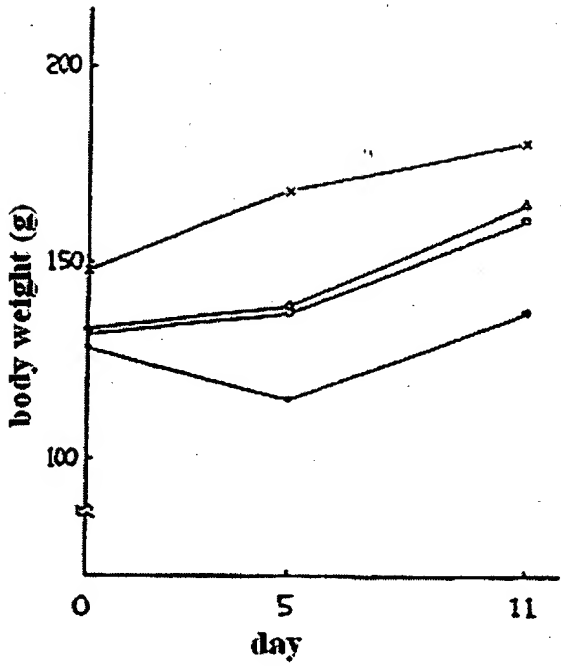


Figure 3